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Use of PC12 Cells and Rat Superior Cervical Ganglion Sympathetic Neurons as Models for Neuroprotective Assays Relevant to Parkinson's Disease

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Abstract

Cellular models composed of primary neuronal cultures or neuron-like cell lines are commonly used to study neuron cell death and to test the neuroprotective properties of specific compounds. Cellular models are easily accessible, permitting dissection and modulation of signaling pathways involved in neuron death. For example, drug or shRNA delivery is more straightforward since there is no blood–brain barrier to cross. However, since these models have their limitations, any important findings should ultimately be verified with animal models and human samples. Here, we describe two cellular models that can be used as a highly informative and easy to use starting point for testing potential neuroprotective drugs for Parkinson's disease: PC12 cells and sympathetic neuronal cell cultures. We describe in detail the protocols needed to apply these models to study neuroprotection in the context of Parkinson's disease.

Keywords

PC12; Parkinson's disease; 6-Hydroxydopamine; 1-Methyl-4-phenylpyridinium; Nerve growth factor

1. Introduction

Tumor-derived cell lines have been extensively used as models to study pathological conditions. The features of such proliferating cells—homogenous and available in large numbers and easy to grow and to transfect or to infect with viral particles—make them highly suitable to screen drugs, manipulate genes, and explore signaling pathways. For neurodegeneration studies, one of the most used and cited culture systems has been the PC12 cell line which was derived from a transplantable rat pheochromocytoma (1). These cells have the machinery to synthesize, release, take up, and store catecholamines, the major species of which is dopamine. A notable feature of PC12 cells is that they respond to nerve growth factor (NGF). In response to NGF, PC12 cells are converted from proliferating chromaffin-like cells to nondividing sympathetic-neuron-like cells that extend axons and become electrically excitable (2, 3). As will be described here, generating and using PC12 cells for neurodegeneration experiments is a relatively simple process.

With respect to their use for studying neurodegeneration, PC12 cells are sensitive to mitochondrial toxins that mimic Parkinson's disease (PD), such as 6-hydroxydopamine (4), 1-methyl-4-phenylpyridinium (MPP^+) (5), rotenone (6, 7), or paraquat (8). These toxins induce cellular degeneration within 24–48 h after exposure. The cells are also reported to undergo degeneration and death in response to overexpression of mutant forms of synuclein that are associated with familial PD (9).

These properties make PC12 an uncomplicated and convenient in vitro model to study causes and possible treatments for PD. However, one must keep in mind that, as with all

models, PC12 cells have their limitations. Any result obtained with this tumor cell line does not assure that the same finding will hold in in vivo models or in the human disease. Therefore, any hypotheses arising from PC12 cell studies should be tested also in primary neuronal cell cultures and in vivo models and, ultimately, wherever possible, in PD itself (10).

One type of primary culture used to study PD is composed of neurons derived from dissociated rodent superior cervical sympathetic ganglia. Like dopaminergic neurons in the substantia nigra, noradrenergic sympathetic neurons of PD patients develop Lewy bodies and undergo degeneration (11, 12). From an experimental point of view, sympathetic neurons have the advantage that they are relatively easy to obtain and culture (13), are a homogeneous population, and also succumb to PD mimetics such as 6-hydroxydopamine (6-OHDA) and MPP^+ (5, 14, 15). They are also useful to assess the role of specific genes by knockdown or by overexpression via transfection or viral infection (13, 16). In addition, they are the primary neuronal counterparts of NGF-treated PC12 cells and as such represent the next step in progressing from experiments in a cell line to cultures of primary neurons.

In this chapter, we will first guide the reader through the process of culturing PC12 cells and sympathetic neurons. We will then describe how to use these cultures to monitor cell viability when testing compounds and treatments for their capacity to protect against PD mimetics.

2. Materials

All work should be carried out in a cell culture hood using sterile technique. It is recommended to disinfect surfaces with 70% ethanol before and after the proceedings.

2.1. Coating Plastic Culture Ware Substrates

1. Rat tail collagen (Roche). Stock solution in 0.2% acetic acid in sterile double distilled or deionized water. Working dilution 1:15 in sterile double distilled or deionized water.

2.2. Coating Glass Coverslip Substrates

1. Poly-D-lysine (Millipore) molecular weight >300 kDa. Dilute stock solution (1 mg/mL) in sterile double distilled water to the final working concentration of 50 μg/mL.
2. Rat tail collagen (Roche). Stock solution in 0.2% acetic acid in sterile double distilled or deionized water. Working dilution 1:15 in sterile double distilled or deionized water.

2.3. PC12 Cells

1. *Complete medium*: RPMI 1640 cell culture medium containing 10% heat inactivated horse serum (Sigma) (see Note 1), 5% fetal bovine serum, and penicillin/streptomycin (50 units/50 μg of each per mL).
2. *Differentiation medium*: RPMI 1640 cell culture medium containing 1% heat-inactivated horse serum and penicillin/streptomycin (50 units/50 μg of each per mL) and 50 ng/mL recombinant human or murine NGF (commercially available from suppliers such as Alomone). This should be added from 1,000× stock just before use.
3. *Freezing medium*: complete medium with 10% dimethyl sulfoxide (DMSO).

2.4. Sympathetic Neuron Cultures

1. Differentiation medium.
2. Uridine/fluorodeoxyuridine: a 500-μM stock solution in sterile double distilled or deionized water.
3. Trypsin (Invitrogen): 0.25% (w/v) in phosphate buffered saline (PBS) (sterile and without EDTA).
4. RPMI 1640 cell culture medium.
5. One tube with 15 mL RPMI 1640 cell culture medium + NGF (50 ng/mL) without serum and other additives.
6. One tube with 25–30 mL of RPMI1640 cell culture medium without serum and other additives.
7. One bucket of ice.
8. Two insulin needles.
9. One polystyrene support for dissection (5×5×1 cm). Wrapping the support in aluminum foil makes sterilization with 70% ethanol much more easier.
10. 70% Ethanol.
11. Two micro dissecting tweezers (Roboz).
12. One dissecting tweezers (Roboz).
13. One dissecting scissors (Roboz).
14. One regular scissors (Roboz).

2.5. PD Toxin Treatments

1. 10-mM 6-OHDA (Tocris) stock in sterile double distilled or deionized water
2. 100-mM MPP^+ (Sigma) stock in sterile double distilled or deionized water

2.6. Viability Assay

1. *10× nuclei buffer* (100 mL): cetyldimethyl-ethanolammonium bromide (5 g), NaCl (0.165 g), glacial acetic acid (2.8 mL), 10% Triton X-100 (50 mL), 1 M $MgCl_2$ (2 mL), 10× PBS (10 mL), and H_2O (35.2 mL). The working dilution is 1× in distilled water (17).
2. Neubauer hemacytometer chamber with coverslip.

3. Methods

We will start by describing how to grow PC12 cells and how to treat them with NGF. We will then discuss how to prepare rat sympathetic neuron cultures. We also provide instructions on how to perform treatments of the cultures with PD mimetic toxins that induce cell death, and finally, we will suggest several options to assess cell viability.

3.1. Growing PC12 Cells (see Note 2)

3.1.1. Coating Plasticware—To coat the plates, pipette the necessary amount of collagen (1:15) to uniformly cover the surface. For a 10-cm culture dish, 1 mL is sufficient, and this volume can be accordingly adjusted for culture dishes/wells of different sizes. Alternatively, one can add a larger volume and then remove the excess with a sterile pipette. Allow the

plates to dry uncovered under a tissue culture hood for at least an hour. After drying, plates can be stored at room temperature, wrapped in aluminum foil or in the initial plastic bags, for 2–3 weeks (see Notes 3 and 4).

3.1.2. Thawing and Plating PC12 Cells

1. If PC12 cells are received or stored in a frozen state (−80°C or in liquid nitrogen), they will be in complete medium and 10% DMSO. Thaw the vial at 37°C in a water bath. This step should be done quickly to diminish the toxicity of DMSO.
2. Prepare a sterile falcon tube with 10 mL of warm (37°C) complete medium.
3. Add the thawed content of the cryotube to the 10 mL of complete medium.
4. Mix well and centrifuge the cells for 5 min at 240×*g* at room temperature in a table top centrifuge.
5. Remove the supernatant (containing DMSO) and add 10 mL of fresh complete medium to resuspend the cell pellet.
6. Mix well by trituration in a pipette and plate the contents in a 10-cm collagen-coated tissue culture plate. This step also serves to break up cell clumps. Shake the plate well, back and forth and left to right, to spread the cells uniformly. Avoid shaking circularly or otherwise the cells will tend to concentrate at the center of the plate.
7. Once the cells are plated, renew the culture medium every 2–3 days. Approximately 2/3 of the medium should be exchanged. Maintain a volume of 5–8 mL of medium.

3.2. Subculturing PC12 Cells

It is recommended to subculture the cells when 90–95% confluence is reached. One plate can be split in two or three plates.

1. Before detaching the cells from the plasticware, aspirate some of the medium from the plate to reach a volume of 3–5 mL. This avoids potential splashing of the medium out of the plate during cell detachment.
2. Incline the plate at about 30°, and with a plastic sterile pipette, eject a few milliliters of medium over the cell monolayer. Cells will detach with the pressure exerted by the medium.
3. After applying medium in this way to the entire surface, triturate the cells several times with the pipette to break up cell clumps. Split the resulting cell suspension into two or three plates (see Notes 5 and 6).
4. Bring the total volume per plate to 5–8 mL of complete medium.

3.3. Freezing PC12 Cells

1. Detach cells from the plates.
2. Centrifuge the cell suspension at 242×*g* for 5 min.
3. Resuspend the pellet from one plate in 2 mL of complete medium containing 10% DMSO.
4. Pipette the suspended cells into cryotubes and place them in an isopropanol-freezing container.

5. Place the container with the cells in a −80°C freezer. The isopropanol container will freeze the cryotubes about 1°C/min (see Note 7). The cryotubes can be stored at −80°C for up to several months and transferred to liquid nitrogen for longer periods of storage.

3.4. Treating PC12 Cells with NGF

PC12 cells dramatically change their phenotype when they are exposed to NGF. They exit the cell cycle, project long neurite-like processes, and take on many properties of differentiated sympathetic neurons, including synthesis, storage, and release of catecholamines (principally dopamine).

1. Detach PC12 cells from the plate as described in Subheading 3.2.
2. Dilute the cell suspension with differentiating medium (RPMI1640, 1% horse serum, and 50 ng/mL NGF). Typically, one confluent culture of PC12 cells can be used to seed 5–20 cultures of the same size for NGF treatment. Note that the NGF stock (50 μg/mL) should be stored at 4°C and should be freshly added to RPMI 1640/1% horse serum medium as needed.
3. Plate the cells on the appropriate collagen-coated plasticware.
4. Exchange ¾ of the culture medium every 2–3 days with differentiating medium plus fresh NGF to a final dilution of 50 ng/mL.
5. The cell should begin to extend neurites within 24 h and will continue to do so over the next 7–10 days.

3.5. Cultivating Sympathetic Neurons from Rat Superior Cervical Ganglia

From one rat litter of 12–14 pups, a 24- or 48-well plate can be obtained. The cell density can be adjusted as desired by counting the cells at the end of the dissection. The steps below describe the preparation of cultures from one litter of newborn rat pups. A video of the dissection and culture procedure can be found at (13).

1. Soak all the dissection tools in 70% ethanol for at least 10 min.
2. Spray or wipe down with 70% ethanol all the surfaces of the dissecting hood, the microscope, and the fiber optic lights. Also, disinfect with 70% ethanol the small polystyrene foam support wrapped in aluminum foil. Put a sterile piece of gauze over the support.
3. Prepare two 10-cm plates with 5 mL of RPMI or PBS. These will be used to rinse the tools during the dissection to wash off any adhering tissue.
4. Place the lids of the 10-cm plates face up in the hood and pipette onto them one or two drops of RPMI for each pup that will be dissected. After dissection, the ganglia will be transferred to these drops for cleaning under the dissecting microscope.
5. Put the tubes with RPMI on ice.
6. Keep the pups in a clean box. Dissect one pup at a time.
7. Spray the pup for dissection with 70% ethanol and immediately decapitate it using a pair of sharpened small dissecting scissors. Remove the head closer to the shoulders than to the jaw so as to avoid cutting through the ganglia.
8. Place the head on the polystyrene support facing up.

9. With the fine scissors, cut the skin of the neck. Pin the skin flaps with the insulin needles to the polystyrene support. This will hold the head in place and prevent the skin from folding over the field of dissection.
10. Pipette RPMI onto the exposed tissue to remove blood cells and debris and to prevent drying.
11. Under the dissecting microscope, with two pairs of fine-tip tweezers, remove the salivary glands and connective tissue.
12. Remove the sternocleidomastoid muscle on the side of dissection to have a better view of the carotid artery. The carotid branches into the external and the internal carotids. The sympathetic ganglion is right beneath the branch point of the artery. With one set of tweezers, pull the lower end of the carotid up, and with the other, remove the translucent tear-shaped ganglion attached to the vessel, that is, the sympathetic ganglion. Close to the external carotid, there is another rounder and smaller translucent ganglion. This is the nodose ganglion. Be careful not to confuse the two ganglia.
13. Put the ganglion in one of the RPMI1640 drops on the culture plate lid.
14. Repeat the dissection to remove the second ganglion on the other side of the neck.
15. Under the dissecting microscope, clean the ganglia by teasing away any remains of the carotid and surrounding connective tissue. This will diminish contamination of the cultures by nonneuronal cells.
16. Transfer the cleaned ganglia to the 15-mL tube with RPMI plus NGF on ice. Collect all ganglia in this way until the dissection of all the pups is finished.
17. Centrifuge the ganglia at 168×*g* for 3 min. Take care because the ganglia tend to attach to the wall of the tube.
18. Remove the supernatant carefully and add 1 mL of 0.25% trypsin in PBS (sterile). Incubate at 37°C for 30 min.
19. To neutralize the trypsin after the 30-min incubation, add 10 mL of RPMI medium with serum. Centrifuge at 168×*g* for 3–5 min, remove the supernatant, and add 2 mL of RPMI with 1% horse serum and 50 ng/mL NGF.
20. Triturate the ganglia for 40–50 strokes in a glass Pasteur pipette with a fire-polished tip. A second Pasteur pipette with a narrower fire-polished tip can be used for the final 15 strokes.
21. Plate the cells (normally two ganglia per well in a 24-well plate) at the desired density, adding more RPMI with 1% horse serum and NGF to bring to the appropriate volume for plating.
22. Twenty-four hours later, add uridine/fluorodeoxyuridine to the wells to reach a final concentration of the antimitotic of 10 μM (see Note 8).
23. Change the medium of the cultures every 2–3 days. Add 10-μM uridine/fluorodeoxyuridine to the fresh medium for the first two medium changes.
24. Use the cultures between 7 and 15 days after plating.

3.6. Toxin Treatments

Both NGF-differentiated PC12 cells and sympathetic neurons will be ready to use after at least 7 days following plating.

1. Prepare stock solutions of the toxins. Use stock concentrations to minimize the volume of solution added to the cultures.
2. Change the medium right before any treatment.
3. If a specific compound is tested in conjunction with the toxins, pretreatment should be done at least 30 min to 1 h before adding the toxin. This is to permit entry into the cells. Always include controls of sister cultures treated with the vehicles used for delivery of toxin or tested agent. If the vehicle is DMSO, the final amount should be no more than 0.1% of the final volume in the culture well.
4. To treat neuronal PC12 cells with (see Notes 9–12):
   - 6-OHDA: a stock of 10 mM is recommended to treat the cells at a final concentration of 50–100 μM.
   - MPP^+: a stock of 100 mM is recommended to treat the cells at a final concentration of 500 μM-1 mM.
5. To treat rat sympathetic neurons, prepare stocks as above, but the final concentrations of the toxins should be much lower: 5–10 μ5M for 6-OHDA and 50–100 μM for MPP^+ (see Notes 9–12).
6. After treatments, wait for the desired time to assess the cultures. If viability is monitored, 24 and/or 48 h are suggested.
7. For analyzing the samples by Western immunoblotting, choose shorter incubation times.

3.7. Viability Assessment

To quantify viable cells, there are several methods to choose from. However, 6-OHDA and MPP^+ with their capability to inhibit mitochondrial complexes can interfere with tetrazolium salt assays, leading to an overestimation of cell death. We routinely use a method with a detergent solution that dissolves the cell plasma membrane and leaves the nuclear membrane intact (17). This permits counting of the numbers of surviving cells in the cultures. With a Neubauer hemacytometer chamber, healthy round nuclei can be counted under a phase microscope and distinguished from the smaller, darker dead or degenerating nuclei.

The procedure is as follows:

1. Aspirate to fully remove the medium.
2. Add 0.5 mL of detergent solution to the wells (in a 48-well plate, add accordingly for other-size dishes or wells) and mix well (see Note 13).
3. Place approximately 10 μL of the nuclear suspension under the coverslip of the Neubauer chamber.
4. Count at least 100 nuclei (see Note 14).
5. Cell survival can be expressed as the percentage of viable cells in experimental cultures compared with sister control cultures.

For transfection experiments, cell viability (after treatment) can be assessed by counting living green fluorescent protein-positive cells in strips under a fluorescence microscope. If the transfection rate is low (as in superior cervical ganglion cultures), counting all the labeled cells in the culture is recommended.

4. Notes

1. If horse serum needs to be inactivated, thaw the bottle and put it in a water bath at 56°C for 30 min.
2. RPMI medium requires an atmosphere enriched in CO_2 to maintain the proper pH. Therefore, an incubator should be used that maintains at 7.5–8% CO_2 atmosphere.
3. Coating glass substrates (such as coverslips or glass slide cultures) for immunofluorescence. To perform confocal microscopy on either neuronal PC12 cells or superior cervical ganglion neurons, it is necessary to culture them on glass. This requires coating the surface with a substrate that permits cell attachment. To coat the glass, first incubate overnight in a solution containing 50 μg/mL of poly-$_D$-lysine. Following this, wash with sterile distilled water, coat with collagen, and let dry as described above.
4. Do not irradiate collagen plates with UV. This damages collagen polymers and decreases cell-binding capability.
5. It is easier to carry out initial experiments (e.g., concentration-response curves) with neuronal PC12 cells, and afterward, to confirm the main results with sympathetic neurons. It is also useful to use 48-well plates to maximize the number of replicate cultures. Routinely, PC12 cells are grown in 10-cm dishes. When using differentiated PC12 cells, the type of plates used for experiments can be adapted to optimize use. For instance, if the experiment is testing viability, it is better to use 24- or 48-well plates to permit multiple (at least 4) replicates. On the other hand, if the object is to collect protein extracts, it is better to use 6-well plates or even 10-cm dishes.
6. PC12 cells tend to detach from the plasticware easily, and even more so, if the plate has been coated with collagen more than 3 weeks earlier. If such detachment starts and the cells are not dying, try to replate them on a fresh collagen coated plate. This problem is common in neuronal PC12 cells after transfection: the older the coating, the more they will detach after transfection.
7. If you do not have an isopropanol container, you can place the cryotubes on ice for 10 min and then freeze them at −80°C.
8. Uridine and deoxyuridine stocks should be prepared and stored in separated tubes.
9. Toxins as 6-OHDA or MPP^+ are generally used to mimic PD in vitro. The dopamine transporter specifically takes up both toxins. Once inside the cells, these toxins deplete catecholamines and inhibit mitochondrial oxidative metabolism. Therefore, their toxicity range will depend on the density of the dopamine transporters on the cells and also on the cellular density itself. For example, identical concentrations of 6-OHDA or MPP^+ will be more toxic in low-density cultures than in more dense ones. The concentrations listed here are intended to cause 40–60% cell death. It is important to work within this range for testing neuroprotective compounds. Also, excess concentrations of the toxins will drive nonapoptotic as well as apoptotic cell death.
10. These toxins inhibit mitochondrial complexes and deplete cells of ATP. In doing so, they also generate damaging reactive oxygen species.
11. Both 6-OHDA and MPP^+ should be freshly prepared just before use. Both are light sensitive, and therefore, their solution tubes should be wrapped in aluminum foil.
12. Always wear gloves when weighing out and working with these toxins.

13. For viability assays that involve counting nuclei, resuspend the contents of the well with a pipette before withdrawing 10 μL for placement in the Neubauer chamber. Nuclei tend to sink to the bottom of the well. Cultures to which the nuclear counting solution has been added can be stored for several days. In this case, seal the plate with Parafilm to prevent evaporation and store it at 4°C for 5 days at maximum.

14. As noted above, at least 100 nuclei should be counted per culture for viability assays. If the numbers of nuclei are excessive, more lysis buffer can be added to dilute the nuclear suspension. Keep track of the volume in final calculations of cell numbers.

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